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14. ABSTRACT:

Our studies show that agonist-bound PR-B can stimulate the proliferation of breast cancer cells by functioning in a direct manner to induce transcription of E2F1 and E2F2, key regulators of cell cycle progression. We demonstrate that although the MAPK pathway is important for phosphorylation of RB and release of E2F, its activation is not dependent on PR signaling through Src family kinases. Further, we found that PRMs such as asoprisnil that do not induce classic PR target genes can activate E2F signaling and stimulate proliferation. Future studies will explore this novel mechanism by which PR regulates breast cancer proliferation so that we can better enable development of PRMs that effectively inhibit breast tumor growth.

15. SUBJECT TERMS
Progesterone receptor, E2F, cell cycle

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Introduction

Progesterone is a naturally occurring steroid hormone that functions by binding to the progesterone receptor (PR) and thereby enabling the receptor to bind DNA, recruit cofactors, and induce the transcription of target genes [1]. In addition, PR indirectly regulates gene expression through a rapid, non-genomic pathway by interacting with Src family kinases and signaling through downstream MAPK [2]. In the breast and other tissues of the female reproductive system, progesterone plays an key role in normal development and function [3]. However, recent data suggests that PR also contributes to the proliferation of breast cancer cells [4, 5]. Given that over half of all breast cancers express PR, antiprogestins which inhibit the proliferative functions of PR may have the potential to block breast cancer progression [6]. Therefore, the goal of this project is to explore the mechanisms by which PR regulates breast cancer proliferation so that we can better enable development of PR modulators (PRMs) that effectively inhibit breast tumor growth. Our studies focus on a novel pathway by which PR may control proliferation of breast cancer cells. In a preliminary microarray study done on T47D breast cancer cells, we found that pre-treatment with the MEK 1/2 inhibitor U0126 altered PR-mediated regulation of a group of over 1000 genes. Promoter analysis revealed that the promoters of a subset of these genes were enriched for E2F binding sites. Since E2F family members are key transcription factors required for regulation of cell cycle, we decided to investigate PR regulation of E2F signaling.

Body

The first task for this project was to define the conditions under which PR positively regulates E2F transcription. In most breast cancer cell lines, estrogens are important for regulation of PR expression; however, estrogen is a known mitogen in breast cancer, and we wanted to concentrate solely on PR-specific regulation of E2F expression and cell cycle. Therefore, we chose the T47D cell line as a model system for our studies because PR functions are uncoupled from ER signaling in this breast cancer cell line. Using real-time qPCR and Western blot analysis, we found that treatment of T47D cells with the synthetic progestin R5020 induces expression of E2F1 and E2F2 at both the mRNA and protein levels (**Figure 1)**. We observed that pre-treatment with the MEK 1/2 inhibitor U0126 blocked R5020-mediated induction of E2F1 and E2F2, indicating that the rapid, non-genomic actions of PR may be partly responsible for its regulation of E2F. In addition, treatment with R5020 induces transcription of downstream E2F target genes such as cdc2 and cdc6.

To ensure that PR is necessary for R5020-mediated induction of E2F transcription, we obtained T47DC42 cells [PR(-) T47D subclone] that stably express a LacZ control, wild-type PR-A, or wild-type PR-B from Dr. Dean Edwards. Real-time qPCR analysis shows that R5020 induces E2F1 and E2F2 mRNA in cells expressing PR-B alone (**Figure 6A)**, but not in cells expressing the LacZ control or PR-A alone (**Figure 2**). Next, we established that regulation of E2F signaling by PR-B is not a unique phenomenon that is restricted to T47D cells. In fact, R5020 induces expression of E2F1 and E2F2 in BT483 breast cancer cells, which have normal coupling of the ER and PR pathways, and in ER-/PR- human mammary epithelial cells (HMECs) infected with a PR-B adenovirus (**Figure 3**).

Our second task was to assess the relative contributions of genomic vs. non-genomic PR actions on E2F signaling. To address whether PR directly regulates the transcription of E2F by binding to its promoter, we performed chromatin immunoprecipitation (ChIP) experiments using synchronized T47D cells treated with vehicle or R5020. ChIP analysis shows that agonist-bound PR is recruited to both the E2F1 and E2F2 promoters, suggesting that PR functions to directly induce E2F transcription (**Figure 4**). However, we have been

unable to identify any classic progesterone response elements (PREs) within the E2F1 or E2F2 promoters. It is feasible that PR associates indirectly with E2F promoters, perhaps through some sort of tethering mechanism. Specifically, since E2F binds to its own promoter in a positive feedback loop, PR might interact directly or indirectly with E2F and consequently be recruited to E2F promoters. Preliminary experiments support this hypothesis by showing that E2F binding sites are required for R5020-mediated induction of E2F2 activity.

Since E2F1 has been shown to bind directly to SRC-3/ACTR, which is a known co-activator of PR, we decided to explore the possibility that agonist-bound PR binds to ACTR, which in turns binds to E2F and is recruited to the E2F promoter. However, an eighty percent knockdown of ACTR expression using siRNA does not affect R5020-mediated induction of E2F1 or E2F2 expression (**Figure 5**). We are in the process of using coimmunoprecipitation experiments and electrophoretic mobility shift assays to verify whether PR associates directly with E2F on the E2F promoter.

Given that R5020-mediated induction of E2F1 and E2F2 is blocked by U0126, we initially thought that the rapid, non-genomic actions of PR may be partly responsible for its regulation of E2F. To further investigate the potential contribution of non-genomic PR signaling to E2F activity, we obtained a T47DC42 PRBmPro cell line from Dr. Dean Edwards that stably expresses a mutant form of PR-B in which three key proline residues in the polyproline motif were replaced with alanines. This mutant PR receptor is unable to mediate rapid, non-genomic activation of Src family kinases or downstream MAPK, but its classical genomic functions remain intact. Contrary to our initial hypothesis, we found that R5020 induces equal expression of E2F1 and E2F2 mRNA in cells expressing wild-type PR-B versus the mutant PRBmPro version (**Figure 6A**). Furthermore, pre-treatment with the Src family kinase inhibitor SU6656 does not abrogate PR-mediated induction of E2F1 or E2F2 mRNA (**Figure 6B**). These data seem to indicate that although the MAPK pathway is important for phosphorylation of RB and release of E2F, its activation is not dependent on PR signaling through Src family kinases.

The final task for this project was to evaluate the E2F-PR axis as a target for therapeutic intervention. We established that PRMs such as asoprisnil that do not induce classic PR target genes can activate E2F signaling and stimulate proliferation (**Figure 7**). Future studies will explore whether PR-regulated E2F activity can be used to accurately predict the proliferative responses of PR agonists, antagonists and SPRMs. Since E2F activity is necessary for cell cycle progression from G_1 to S phase, we expect to find that PRMs which induce breast cancer cell proliferation also positively regulate E2F activity. If this proves correct, then E2F activity could potentially be used as a screen to identify novel PRMs that inhibit the proliferation of hormone-dependent cancers.

Key Research Accomplishments

- Agonist-bound PR-B can stimulate the proliferation of breast cancer cells by functioning in a direct manner to induce transcription of E2F1 and E2F2.
- Although the MAPK pathway is important for phosphorylation of RB and release of E2F, its activation is not dependent on PR signaling through Src family kinases.

Reportable Outcomes

Therapeutic Implications of Progesterone Receptor-Mediated Regulation of Cell Cycle in Breast Cancer. Ogden HE and DP McDonnell. Poster presented at Duke University Medical Center Department of Pharmacology and Cancer Biology Annual Retreat, Wrightsville Beach, NC (September 29-30, 2006).

Conclusion

Our studies show that agonist-bound PR-B can stimulate the proliferation of breast cancer cells by functioning in a direct manner to induce transcription of E2F1 and E2F2, key regulators of cell cycle progression. We demonstrate that although the MAPK pathway is important for phosphorylation of RB and release of E2F, its activation is not dependent on PR signaling through Src family kinases. Further, we found that PRMs which do not induce classic PR target genes can activate E2F signaling and stimulate proliferation. Future studies will explore this novel mechanism by which PR regulates breast cancer proliferation so that we can better enable development of PRMs that effectively inhibit breast tumor growth.

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4. Rossouw, J. E. et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama 288, 321-33 (2002).
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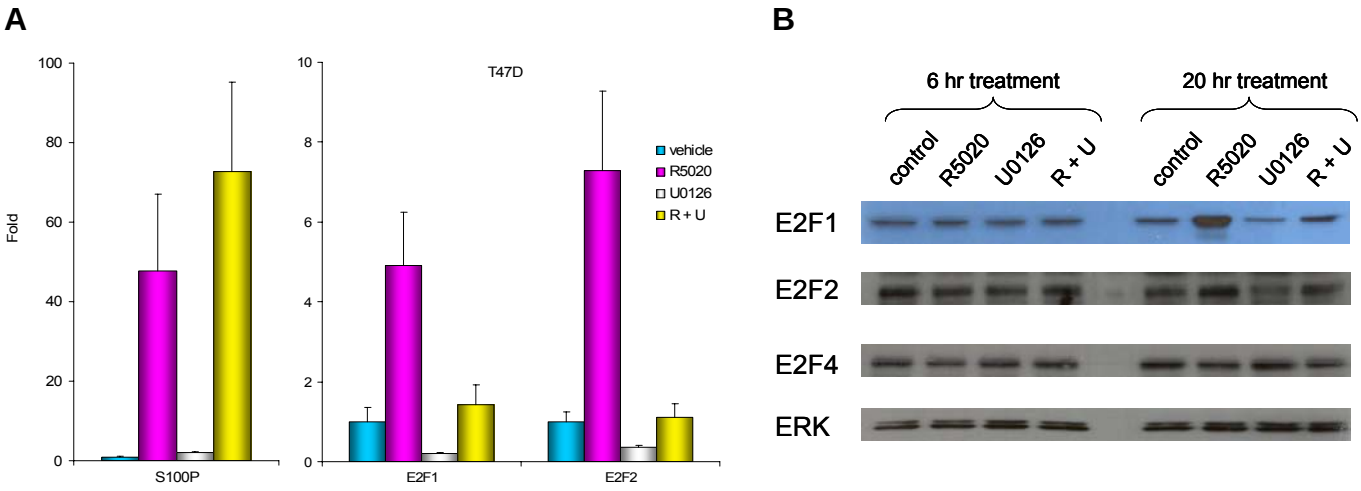
Appendices

None.

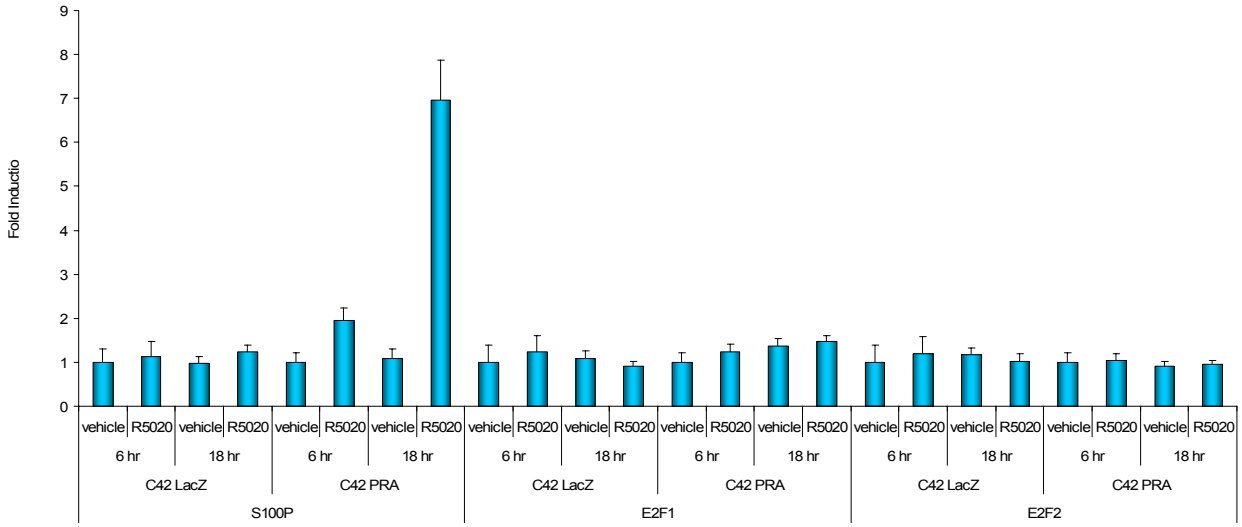
Supporting Data

Figure 1. R5020 induces expression of endogenous E2F1 and E2F2 mRNA and protein in T47D breast cancer cells.



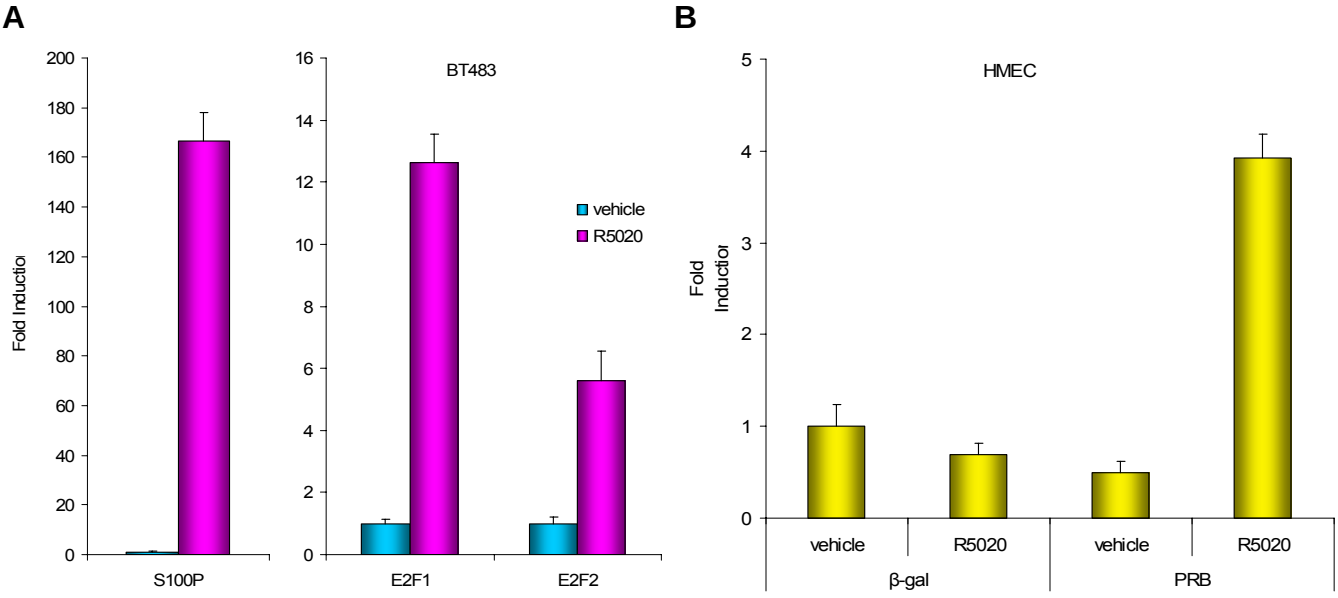
Synchronized T47D cells were treated with vehicle or 10 nM R5020 +/- 10 μM U0126 for various time points and harvested. (A) Real-time qPCR and (B) Western blot analysis show that R5020-mediated induction of E2F1 and E2F2 mRNA and protein levels in T47D cells is repressed by U0126.

Figure 2. R5020 does not induce E2F1 or E2F2 mRNA in PR- T47DC42 cells expressing LacZ or PRA alone.



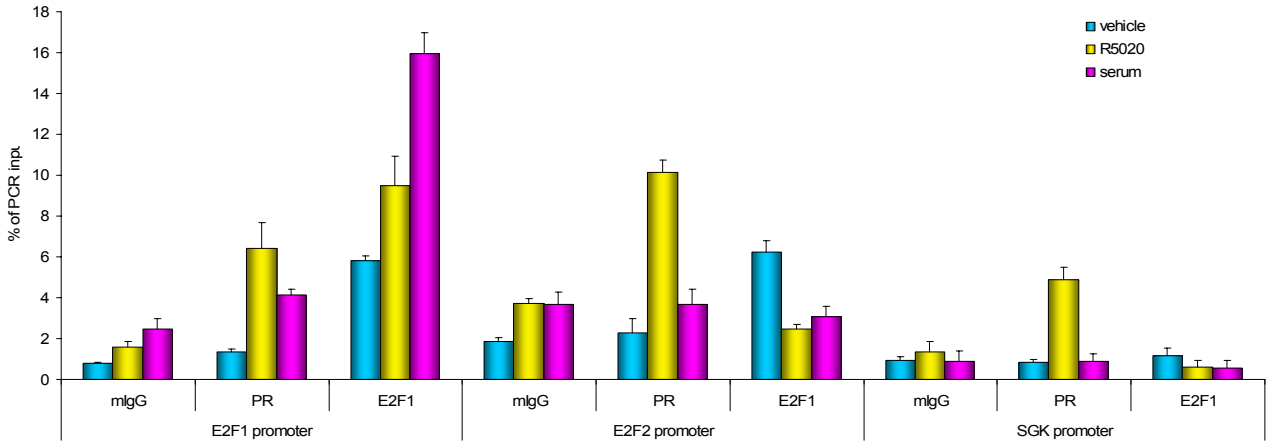
Synchronized T47DC42 cells were treated with vehicle or 10 nM R5020 for 6 or 18 hours, and harvested. Real-time qPCR analysis shows that R5020 is unable to mediate induction of E2F1 or E2F2 mRNA in T47DC42 cells lacking PRB.

Figure 3. R5020 induces E2F1 or E2F2 mRNA in BT483 breast cancer cells and in HMECs infected with a PR-B adenovirus.



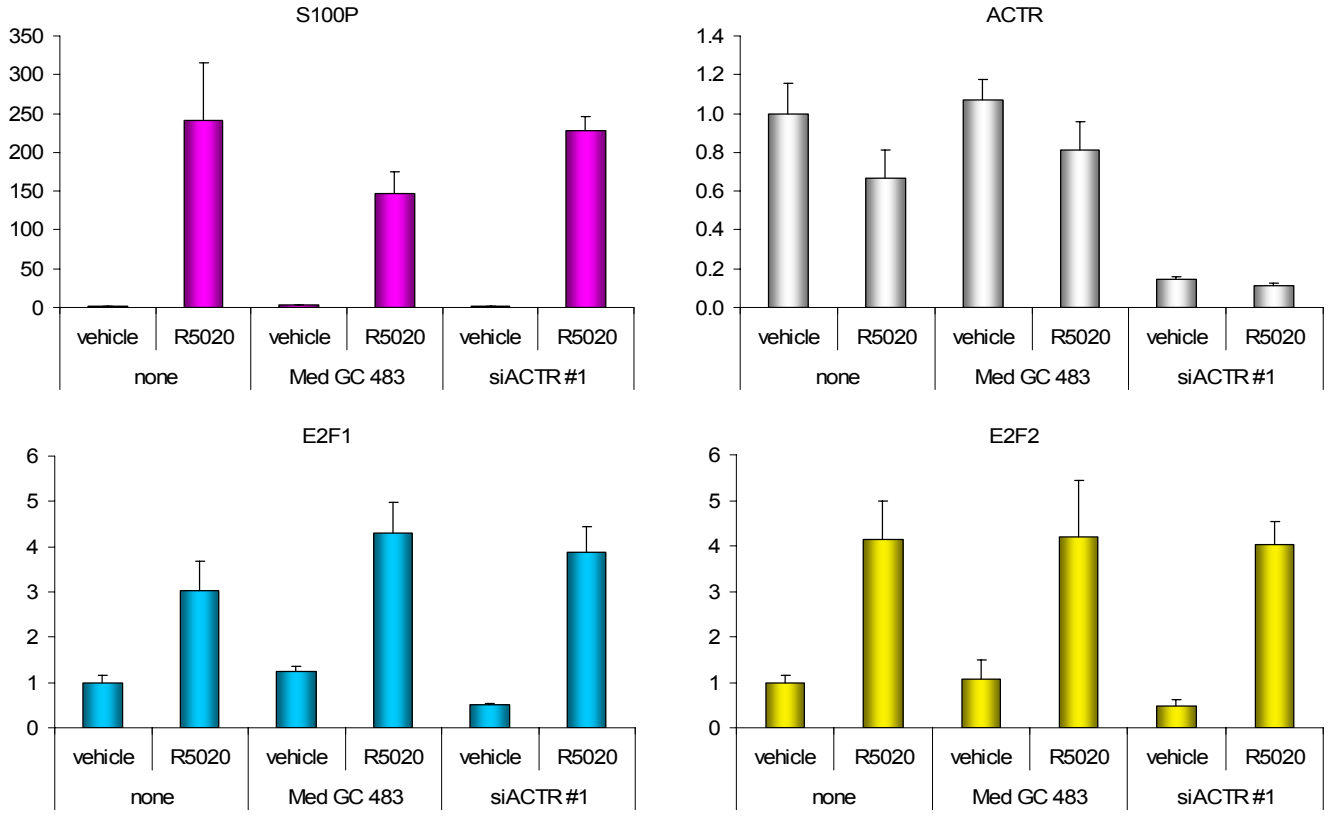
(A) BT483 breast cancer cells and (B) HMECs infected with a control β-gal or PR-B adenovirus were treated with vehicle or 10 nM R5020 for 18 hours. Real-time qPCR analysis shows that R5020 mediates induction of E2F1 and E2F2 mRNA levels in both cell lines.

Figure 4. PR is recruited to the E2F1 and E2F2 promoters in the presence of R5020.



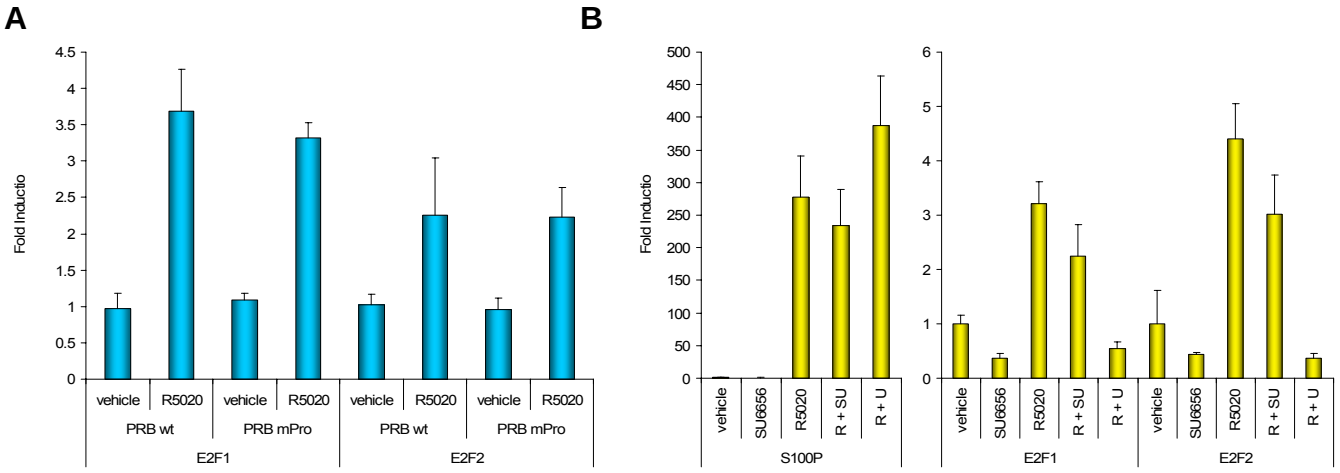
T47D cells were treated with vehicle, 10 nM R5020, or 15% FBS for 60 minutes and subjected to ChIP analysis. Cross-linked chromatin fragments were immunoprecipitated with a PR- or E2F1-specific antibody and analyzed using real-time qPCR.

Figure 5. Knockdown of ACTR does not abrogate R5020-mediated induction of E2F1 or E2F2 expression.



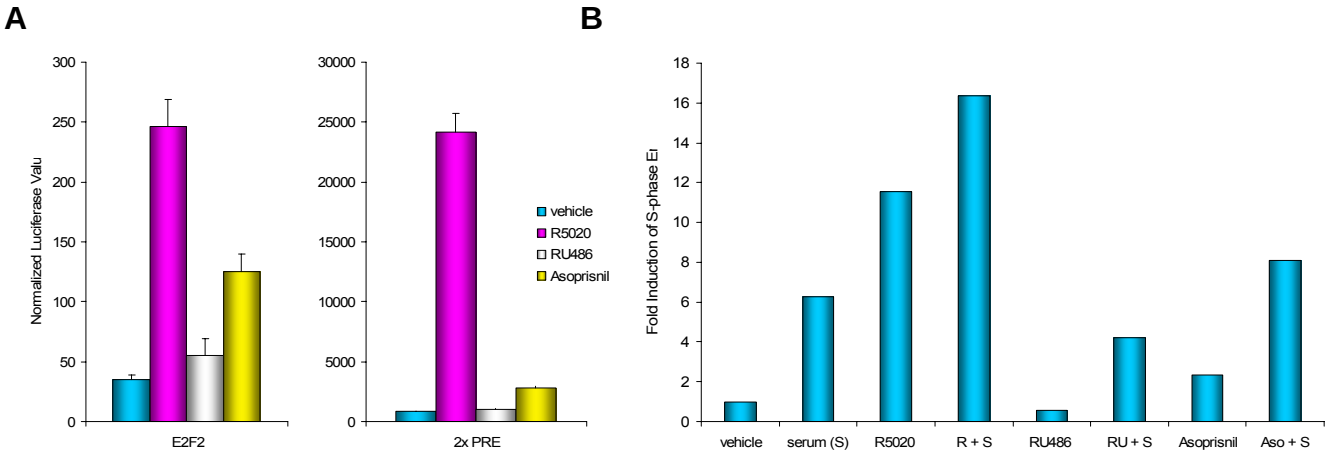
T47D cells were transfected with control siRNA (Med GC 483) or siACTR for 24 hr and subsequently treated with vehicle or 10 nM R5020 for 18 hours. Real-time qPCR analysis shows that an 80% knockdown of ACTR does not affect R5020-mediated induction of E2F1 or E2F2 expression.

Figure 6. PR signaling through Src family kinases is not required for R5020-mediated induction of E2F expression.



(A) T47DC42 cells were treated with vehicle or 10 nM R5020 for 18 hours and harvested. Real-time qPCR analysis shows that R5020 induces similar E2F1/E2F2 mRNA levels in cells expressing wild-type PR-B vs. the mutant PRBmPro version. (B) T47D cells were treated with vehicle or 10 nM R5020 +/- 1 μM SU6656 or 10 μM U0126 for 18 hours and harvested. Real-time qPCR analysis shows that R5020-mediated induction of E2F1/E2F2 mRNA levels in T47D cells is not abrogated by SU6656.

Figure 7. PRMs that do not induce classic PR target genes may activate E2F signaling and stimulate proliferation.



(A) T47D cells were transiently transfected with hE2F2-luc or 2xPRE reporter constructs, treated with vehicle, R5020, RU486 or asoprisnil for 24 hours, and assayed for luciferase activity. (B) Synchronized T47D cells were treated with various ligands for 16 hours. S-phase entry was measured during a 4 hour BrdU incorporation assay.